The Origin and Formation of Fibrous Tissue Produced as a Reaction to Injury in Pecten Maximus, as a type of the Lamellibranchiata.

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With Plate 24.

Contents.

Introduction.

The experiments described in this paper were performed on Pecten maximus at the Laboratory of the Marine Biological Association at Plymouth.

The object of our work was to investigate the histology of the reaction of the tissues to the presence of a foreign body, and to determine the origin and method of formation of the fibrous tissue formed around it.

As one type of foreign body we chose sterile agar jelly, which has little or no irritative or toxic action on the tissues, and is not removed by phagocytosis. As another type we chose masses of gill-tissue and of the tissue of the digestive gland, taken from an animal of the same species. Neither of these could be injected under aseptic conditions, and both were capable of removal by phagocytosis. Considerable irritation was set up by the implantation of these tissues, especially in the case of the digestive gland. This produced marked degeneration of the neighbouring tissues, possibly owing to the liberation of ferments and consequent digestive action.

Pecten maximus was selected for these experiments on account of the large size of its adductor muscle, which presents a homogeneous mass of tissue particularly suitable as a site for implantation of foreign bodies. Before making this choice, experiments were tried on several other animals, but it was found that in most cases the technical difficulties encountered in endeavouring to make implantations into small masses of tissue, and in determining the exact relation of the underlying organs to the superficial anatomy, were too great to render these animals suitable subjects for experiment.

Such experiments were tried on Carcinus mænas, Pagurus bernhardus, and others of the smaller species of crabs, on Palæmon serratus, Ligea oceanica, Aphrodite aculeata, Patella vulgata, Aplysia punctata, Archidoris tuberculata, and many Lamellibranchs, but none offered such promise of success as Pecten maximus.

Methods.

Pecten maximus can be readily obtained in the Salcombe Estuary. It was found necessary to allow these animals to become acclimatised to living in the laboratory tanks before proceeding to the experimental work. When first placed in the tanks the mortality was heavy, often amounting to 30

per cent. in the first three days, but after the lapse of about a week the survivors appeared to be fully acclimatised to the changed conditions, and often remained healthy for some months.

Experiments on animals whose health was doubtful were of no value, both because the shock consequent on the injection of the foreign body frequently caused death, and also because the reaction of the tissues was not normal in unhealthy specimens. When a Pecten is healthy it lies with the valves of the shell slightly apart, the tentacles are expanded, and it responds rapidly to any stimulus by closing the shell; when held up in the air, the water which drains away is clear and contains no slime. An unhealthy specimen lies with the valves of the shell wide open, there is little or no response to stimuli, and the valves only close under pressure. The tentacles are retracted, and the gonads, gills, and tissues generally, look flabby and unhealthy. The water which flows out between the valves is slimy and viscid, and this is generally the first sign of deterioration.

All instruments used in the experiments were carefully sterilised in boiling water.

The transplanting needle resembles a large hypodermic needle about 1 mm. in diameter and 6 cm. long. Into the hollow needle a somewhat longer stylet fits closely and works like a piston. Any material taken up in the point of the needle is sucked in by drawing the stylet back, and again ejected by pushing it forward.

For injecting into the muscle, a solution of agar in sea-water, coloured by a little hæmatein, was used. The agar jelly was liquefied by heating in boiling water, and was drawn up into the transplantation needle. On cooling it forms a cylinder, of the diameter of the needle, which is easily introduced into the muscle.

The adductor muscle of Pecten maximus is so large that there is no difficulty in selecting a spot at which to bore the shell. The apex of an equilateral triangle, having for its base the line of junction of the posterior auricula with the

right valve, marks roughly on the surface a point at which the shell may be bored without damage to any organ. But as the animal gapes when removed from its tank, it is easy to slip a cork between the valves and select a spot by inspection.

The holes were drilled in the convex or right valve by an ordinary dentist's drill, the head of which was prevented from penetrating too deep by a lapping of thread.

The spot selected for drilling was sterilised with a saturated solution of corrosive sublimate, washed off with a solution of hydrogen peroxide (30 vols.) or distilled water, care being taken not to allow any of the sublimate to run between the valves. The transplanting needle was then introduced to the required depth, slightly withdrawn, and its charge projected into the channel. The hole was then thoroughly dried, and stopped with sealing-wax. If the drying is thorough the wax will adhere after the animal has been returned to the tank. It would, of course, have been possible to implant directly into the muscle through the opening of the valves, but the risks of sepsis would have been greater.

When required for examination, the shell was opened by cutting the adductor muscle at its attachment to the right or convex valve, and a portion of the muscle containing the implanted material removed. This was fixed by three or four hours' immersion in Gilson's fluid, then thoroughly washed, passed through the alcohols, cleaned in xylol, and embedded in paraffin wax. It was then cut into serial sections eight μ thick.

Delafield's hæmatoxylin, followed by Van Gieson's stain, or Benda's iron mordant and hæmatoxylin were used for staining.

Description of the Tissues of Pecten maximus involved in the Experiments, and the Normal Process of the "Clotting" of the Blood.

The adductor muscle of Pecten maximus consists of two portions, bound together by the same sheath of connec-

tive tissue, but differing in structure. The larger, semi-transparent and whitish, consists of striated fibres. The fibres of the smaller, which is opaque and dead white, and lies against the posterior surface of the larger mass, are non-striated. It was into the larger mass that all material in our experiments was introduced.

There is a large blood supply to the muscle from the adductor artery (Dakin, 2), and it contains numerous lacunar spaces. Scattered through it are numerous strands of connective tissue. These contain fibroblasts with deep staining nuclei and long fibrillar processes.

The digestive gland has a tubular structure and completely surrounds the stomach, into which its ducts open. The ducts break up into numerous alveoli, which ramify and ultimately form cæca. The ducts are lined with ciliated epithelium, and the alveoli with secreting cells. These secreting cells are said to degenerate and become filled with a granular pigment, and are ultimately shed into the lumen of the ducts (Dakin, 2). Thus in their younger stages they appear to have a secretory, and in their later stages an excretory function. In addition to these glandular cells, fibrous connective tissue and unstriated muscle-fibre are present. The ducts contain particles of food material, algæ, diatoms, and bacteria, and consequently as a rule septic conditions prevail in the experiments.

The blood of Pecten maximus is a slightly cloudy, colourless fluid. It does not coagulate, but when shaken a number of small, white, flocculer masses appear, which soon fall to the bottom of the tube, leaving the supernatant fluid clear and transparent. These masses consist of blood-corpuscles agglutinated to form plasmodia.

The corpuscles, although varying in size, appear to be only of one kind. They are amœboid bodies, which when expanded protrude a number of slender pseudopodia. When contracted, they are ovoid or spherical. There is a single compact nucleus, staining readily with methylene-blue. The cytoplasm is finely granular, and stains with eosin, but there are

no large eosinophile granules. According to Cuénot (1), they originate in a "glande lymphatique" situated at the base of the gills.

One of us (Drew, 4) has shown in the case of Cardium norvegicum that when the corpuscles come in contact with a rough foreign body, or with injured tissue, they possess the power of agglutinating and forming a compact plasmodial mass. In this way bleeding from a small wound is stopped. When the edges of a wound are covered with this mass of agglutinated corpuscles, protoplasmic strands are formed across the wound, connecting the plasmodia; these strands thicken and contract and so approximate the edges of the wound. So far as our observations go, there is no reason to suppose that the blood of Pecten maximus differs in any of these particulars from that of Cardium norvegicum.

That Lamellibranch blood-corpuscles are capable of a phagocytic action towards degenerated cells has been shown by De Bruyne (3) in the case of Mytilus edulis, Ostrea edulis, Unio pictorum, and Anodonta cygnæa. Sir Ray Lankester (5 and 6) has shown that certain corpuscles of Ostrea edulis have a phagocytic action on diatoms and minute green algæ, and it has been shown by Drew (4) that the corpuscles of Cardium norvegicum have a phagocytic action on bacteria, and are attracted towards extracts of dead tissues.

The Formation of Fibrous Tissue in the Site of the Implantation of a Mass of Gill-Tissue.

As bacteria are normally present on the gill-filaments, the conditions when gill-tissue is implanted differ totally from those obtaining when sterile agar is used.

The implantation soon produces an intense inflammatory reaction on the part of the animal. The blood-spaces in the immediate neighbourhood of the implanted tissue become distended and crowded with corpuscles, which escape from the lacunar spaces and migrate towards the source of irrita-

tion, travelling in all directions between the muscle-fibres. On reaching the gill-tissue the corpuscles come to rest, and form a dense, agglutinated, plasmodial mass, completely surrounding and shutting off the gill-tissue from the neighbouring muscle (fig. 1). They soon appear as if they had undergone some degree of pressure and the nuclei are slightly flattened, probably owing to the contraction of the plasmodial mass as it tightens round the implanted gill-tissue (Drew, 4). In time the corpuscles show signs of degeneration; the nuclei become irregular in outline, and the chromatin is represented by numerous granules staining darkly with hæmatoxylin. The degenerated mass of corpuscles is then invaded by fresh blood-cells, and is more or less completely removed, apparently partly by a process of phagocytosis and partly by autolysis.

While this is going on, the cells of the gill-filaments have degenerated, their outlines are ill-defined, and the nuclei no longer discernible; the bacteria present multiply considerably.

The degenerated gill-tissue is then invaded by blood-corpuscles which have penetrated through the surrounding mass of agglutinated cells, and in most cases the bacteria and epithelial débris are removed by phagocytosis, leaving only the chitinous supporting-rods of the gills.

In the course of this process many of the invading cells also are destroyed, and appear in their turn to be removed by other phagocytes. In time the whole space originally occupied by the gill-tissue becomes filled with a loosely packed mass of blood-cells, among which the chitinous supporting bars are the only relics of the original implanted mass. In many of our experiments bacteria multiplied so rapidly that the phagocytes were unable to cope with them. Consequently the bacteria invaded the neighbouring tissues, entered the blood-spaces, and rapidly caused death.

In preparations from obviously unhealthy animals, it was commonly found that the bacteria had penetrated beyond the protecting mass of agglutinated cells and had invaded the

muscular tissue, which showed signs of degeneration in its somewhat swollen fibres and faint striation.

When a blood-space had been entered, bacteria were often seen ingested by the blood-corpuscles, but in later stages it was obvious that the number of bacteria was so out of proportion to the number of corpuscles that they could not all be removed by phagocytosis, and were of necessity distributed all over the body in the blood-stream.

During these processes the fibroblasts in the walls of the blood-spaces, and in the intermuscular connective tissue in the neighbourhood of the implanted mass, undergo rapid division. This rapid division, resulting from the reaction of the tissues to the irritation caused by implantation, appears to be always amitotic. Mitotic division was only observed in much later stages, when the source of irritation had been removed by phagocytosis, and the rate of division of the fibroblasts was much slower.

Before amitotic division the fibroblasts lose their spindle shape and become oval; a split then appears at one end, and progresses in the plane of the long axis of the nucleus until two daughter nuclei are formed, attached to each other at one extremity, and inclined at an acute angle to one another. These gradually straighten out until they form an hour-glass-shaped mass of nuclear material. Finally the two nuclei are separated at the constriction and become almost circular in shape.

As a result of this active multiplication of the fibroblasts, the strands of connective tissue bounding the blood-spaces and forming the intermuscular connective tissue become crowded with nuclei. The bodies of the fibroblast cells become very indistinct, and little beyond rows of elongated nuclei is discernible. As the multiplication becomes more rapid the typical spindle shape of the nuclei is lost, and they become first oval and finally circular.

There appears to be a constant migration of these cells, with round and oval nuclei, towards the site of implantation. They have very little cytoplasm, and from this, and their

smaller size, are easily distinguished from the blood-corpuscles (figs. 2 and 3). These fibroblasts largely follow the course of the strands of fibrous tissue bounding the blood-spaces, and they appear to travel along in the spaces, being most plentiful near the walls. At the same time, when they multiply very rapidly, many migrate in all directions between the muscular fibres towards the implanted tissue, and are not confined to travelling only in the proximity of pre-existing connective-tissue strands.

On reaching the degenerating layer of agglutinated corpuscles surrounding the implanted tissue, they arrange themselves in rows, and their nuclei elongate in such a direction that their long axes form arcs of a circle surrounding the implanted tissue. Some fibroblasts penetrate among the degenerating cells of the gill-tissue, which are being removed by phagocytes, and in this position start the formation of fibrous tissue.

The surrounding layer of fibroblasts gradually thickens, and presents a somewhat stratified appearance. At first this layer contains a number of blood-corpuscles, but these eventually are removed, probably by autolysis, leaving only the fibroblasts, which can now be seen to be connected with each other by numerous fine processes of the cytoplasm, the whole presenting a somewhat reticulated appearance. In time this tissue becomes more compact, and the reticulation vanishes. It would appear that this has been caused by the contraction of the processes of the fibroblasts, with consequent approximation of the cells. Finally, the nuclei become long and spindle-shaped, the amount of cytoplasm slightly increases, and a layer resembling normal fibrous tissue results.

In our experiments the great variation in the rapidity with which the various changes described took place was very noticeable. The health of the animal after the experiment seems an important factor in accounting for this, for the slow rate of fibrous tissue formation in unhealthy, as compared with healthy animals, was very marked.

Unfortunately none of the animals into which gill-tissue

was implanted lived long enough for all the elements of the gill-tissue to be completely replaced by fibrous tissue, but in healthy specimens most of the signs of inflammation had vanished, and the implanted tissue was surrounded by a wall of apparently healthy fibrous tissue, in four or five days.

Formation of Fibrous Tissue around the site of implanted Digestive Gland Cells.

After the implantation of portions of the digestive gland, a marked degeneration of the muscular fibres in its neighbourhood is noticeable. They swell slightly, all trace of striation is soon lost, and they stain less intensely. The area of degeneration gradually extends, and the muscular fibres in the immediate neighbourhood of the gland tissue are slowly dissolved. This action is presumably due to the presence of ferments in the digestive gland, which digest and render soluble all tissues in the immediate neighbourhood. At the same time the cells of the gland itself degenerate and appear to undergo auto-digestion, so that eventually only the brown pigment-granules originally contained within the secreting cells remain. Under these conditions bacteria do not seem to multiply, though they must have access to the cæca of the digestive gland, as these are in direct communication with the alimentary canal. In none of our sections have we been able to find bacteria, though it is quite common to find the siliceous skeletons of diatoms in the cæca. It seems, therefore, probable that the presence of digestive ferments inhibits the multiplication of bacteria.

As a result of the implantation of this tissue a condition of intense inflammation is set up, and all the blood-spaces in the neighbourhood become distended with blood-corpuscles. There appears to be an endeavour on the part of the organism to shut off all the implanted gland, together with the area of muscular tissue which has undergone degeneration, from the general blood-stream. This is effected by the formation of a layer of agglutinated blood-corpuscles around

the whole of the affected area (fig. 4). It was very noticeable in our preparations that the degenerated area was always larger in specimens that had been implanted with the digestive gland for some time (up to six days), than in those implanted for shorter periods, and thus it would seem that the range of action of the digestive ferments gradually increases. The degenerated area was always found surrounded by a layer of agglutinated corpuscles, though in different specimens this layer varied considerably in thickness. It would seem that while the degenerative process is spreading the layers of corpuscles must be continually dissolved, and others formed a little further back by the spread of the digestive ferments. During this process the fibroblasts undergo division as in the case of the gill-tissue, but while the inflammation is much more acute, the multiplication of fibroblasts is not so rapid, and they are not nearly so noticeable a feature in the sections. In the form of rounded cells, with oval or spherical nuclei, they migrate in small numbers towards the layer of agglutinated blood-corpuscles. Here they share the fate of the corpuscles, being dissolved by the digestive ferments, and accordingly there is no formation of fibrous tissue.

We were never able to keep the animals alive for more than six days. At the end of this time all that remained of the digestive gland was the brown pigment-granules and a little epithelial débris. This was surrounded by a space from which most of the muscular tissue had been dissolved, and this again by a relatively large area of degenerated muscle-fibres. Finally, the whole was surrounded by a layer of agglutinated blood-corpuscles, into which a few fibroblasts were making their way.

These experiments show that the protective layer of corpuscles must very completely shut off the space it encloses from the neighbouring tissue. If this were not the case the digestive ferments, once they had gained access to the blood, would rapidly become disseminated over the whole body. Instead of this, we have distinct evidence that there is a slow and

steady invasion of the tissues by the ferments, and that the area of their action is always contained within a protective layer of agglutinated blood-corpuscles. It seems probable that the digestive gland, when implanted, contains little or no free enzyme, and quickly becomes surrounded by the protective layer of corpuscles, and that later the enzymes are slowly evolved from the zymogens contained within the cell. The vitality of these cells has been impaired by removal from their normal connections and by implantation into the muscle tissue, and accordingly they are dissolved by the enzymes they have themselves evolved.

The Reaction of the Tissues to Implanted Agar Jelly.

Sterile agar jelly has no irritative action on the muscle, and so differs from the tissues previously described.

Agar jelly may be regarded as a physiologically inert substance, and as in these experiments it was made from sea-water in which the Pecten were living, it was approximately of the same salinity as their blood (Dakin, 2), and so was of the same osmotic concentration. Further, the cylindrical rods of agar are remarkably smooth, and if unbroken present no rough surface, except possibly at the extremities.

One of us (Drew, 4) has shown that in the case of Cardium norvegicum, the agglutination of the blood-corpuscles (*in vitro*) is much influenced by the nature of the substance on which they impinge, and that it occurs very much more readily when they come in contact with a rough surface from which a large number of small points may be imagined to project, than when they impinge on a smooth, polished body. It seems probable that similar conditions obtain in the case of the blood of Pecten maximus.

In accordance with these properties of the agar jelly, it was found that absolutely no inflammation resulted from its implantation in the muscle. No layer of agglutinated corpuscles was formed round it, and there was no sign of the collection of unusual numbers of the corpuscles in the

vicinity, nor of any distension of the blood-spaces. The fact that the rod of jelly was always implanted as far as possible parallel to the long axes of the muscle-fibres, and that they were usually rather separated from each other, than cut by the insertion of the transplanting needle, probably contributed towards this result.

After a period of about seven to eight days there were signs of division of the fibroblasts in the neighbourhood of the implanted mass, and a slow migration of the new-formed cells towards the agar took place. By about the tenth day these cells had arranged themselves so as to form a thin and delicate ensheathing layer. The process presents marked differences from that which occurs after the implantation of a substance which causes an inflammatory reaction, with the consequent development of a protecting layer of agglutinated corpuscles. The division of the fibroblasts, instead of being rapid and amitotic, is comparatively slow, and frequently, though not always, mitotic. The nuclei of the young fibroblasts retain their elongated shape, and though the nuclei of the dividing cells lose their typical spindle-like appearance and become oval, they do not become round, as in the case of rapid division after inflammation. The layer of fibrous tissue formed is thinner and less compact, the proportion of cytoplasm to nucleoplasm is greater, and the nuclei assume their typical spindle shape more rapidly. The process seems to be complete by the tenth day, and the appearance is almost identical with that shown in fig. 5, which represents the condition after seventeen days.

In some of our experiments the sealing-wax with which the drill holes were closed became detached in the tank. The holes were re-sealed as soon as this was noticed, but the animals seldom survived long. On sectioning, an area of inflammation was usually found surrounding the agar, and rapid division of the fibroblasts in the vicinity was in progress. In specimens that survived longer a complete sheath of fibrous tissue had formed round the agar, and the condition resembled that resulting from implantation of gill-

tissue. It seems that in these cases bacteria must have entered through the drill-hole, and, travelling between the agar and muscle, have caused an inflammatory reaction. In one other case, in which the hole had not come unsealed, inflammation and formation of fibrous tissue occurred, but as this only took place once out of twenty-six implantations made with sterile agar, it is probable either that the sealing-wax plug leaked at the edges or that bacteria found their way in when the agar was introduced.

Summary of Results.

Our experiments show that the implantation of a tissue, such as that forming the gills, accompanied by the bacteria which adhere to it, produces an intense inflammatory reaction. This is characterised by the active migration of blood-corpuscles, which form a plasmodial mass around the implanted tissue, shutting it off from the general circulation. This protective layer is gradually removed by phagocytosis and autolysis, and at the same time the gill-tissue is invaded and removed by phagocytes. While this is going on, rapid amitotic division of the fibroblasts in the neighbourhood occurs; they lose the typical spindle-shape of the nuclei, and the new-formed cells consist of rounded or oval nuclei, with a scarcely perceptible amount of cytoplasm. These rounded cells migrate towards the implanted tissue, and arrange themselves in layers around it, the nuclei become elongated, and the proportion of cytoplasm increases. Finally, a layer of typical "scar" fibrous tissue is formed, enclosing the chitinous skeletons of the gill-bars.

In the case of the implantation of digestive gland tissue a similar protective layer of agglutinated corpuscles is formed, but this is continually dissolved up and reformed, as the sphere of action of the enzymes in the cells of the digestive gland extends. All the muscle-fibres within this protective layer soon lose their striation, swell, and are partially dissolved, presumably by the digestive enzymes. The fact that

there is a progressive extension of this digestive action shows that the layer of agglutinated corpuscles performs its protective function very completely, as otherwise the enzymes would escape into the general circulation. Simultaneously the fibroblasts in the vicinity multiply and migrate, as in the case of implanted gill-tissue, but the multiplication does not seem to be so rapid. No permanent layer of fibrous tissue is formed, as the migrated fibroblasts are dissolved in the course of the extension of the sphere of action of the digestive ferments.

In the case of the implantation of sterile agar jelly, made with sea-water, no inflammation results, and for some time there is no sign of any reaction of the tissues if absolute asepsis has been ensured. After seven or eight days there is a slow and often mitotic division of the neighbouring fibroblasts; they migrate and rearrange themselves to form a thin layer of fibrous tissue around the agar.

It is noteworthy that though the tissues and the blood, especially in its manner of forming a "clot," present marked differences from those in Vertebrates, yet the formation of fibrous tissue, as a reaction to injury, does not differ in any essentials from the process which takes place in the higher types.

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DESCRIPTION OF PLATE 24,

Illustrating the paper by Messrs. G. H. Drew and W. de Morgan on "The Origin and Formation of Fibrous Tissue produced as a Reaction to Injury in Pecten maximus, as a type of the Lamellibranchiata."

Reference Letters.

ag. Agar. *agg. lyr.* Agglutinated layer of blood-corpuscles. *b.c.* Blood-corpuscles. *deg. gill.* Degenerated gill-tissue. *deg. msl.* Degenerated muscle. *dig. gl.* Digestive gland-tissue. *div. fbl.* Dividing fibroblasts. *fbl. lyr.* Fibroblast layer. *mig. fbl.* Migrating fibroblasts. *msl. fbr.* Muscle-fibres.

[N.B.—In the figures the bundles of muscle-fibres are shown as a whole: the individual fibrils and their striations are not differentiated. The size of the muscle-bundles differs considerably in different parts of the adductor muscle.]

Fig. 1.—× 400. Gill-tissue which has been implanted for sixteen hours. A layer of agglutinated corpuscles divides the degenerated gill-tissue on the left from the muscular tissue on the right. Corpuscles are making their way between the muscle-fibres to join the agglutinated layer.

Fig. 2.—× 300. A later stage of fig. 1, taken seventy-two hours after implantation. A definite layer of fibrous tissue has been formed round the gill-tissue, which is completely degenerated and invaded by phagocytes. The fibroblasts are dividing and migrating towards the lesion.

Fig. 3.—× 700. A more highly magnified portion of one of the blood-spaces drawn from the same section as fig. 2. The fibroblasts are undergoing amitotic division, and migrating towards the gill-tissue, where they arrange themselves to form a layer of fibrous tissue.

Fig. 4.—× 450. Digestive gland-cells (on the left) which have been implanted for ninety-six hours. External to them is a region of degenerated and partially dissolved muscle-fibres, which is divided from the normal muscle by a thin layer of agglutinated corpuscles. These are also rapidly degenerating, but are reinforced by the continued arrival of fresh corpuscles. The cellular structure of the alveoli of the digestive gland has been lost, leaving little beyond traces of the original cell walls and the brown pigment-granules.

Fig. 5.—× 450. Agar jelly (to the left) which has been implanted for seventeen days. It is divided from the muscle-tissue by a delicate layer of fibroblasts.